Supplemental Data

Generation of Induced Pluripotent Stem Cells using Recombinant Proteins

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Supplemental Experimental Procedures

Plasmids construction

Codons of human *Pou5f1/Oct4* (NP_002692), *Sox2* (NP_003097), *Klf4* (NP_004226), and *cMyc* (NP_002458) were first optimized for high level protein expression in *E. coli*. They were then synthesized using DNA oligo based, PCR gene assembling method. Poly-arginine tag plus a linker sequence, *i.e.* ESGGGGSPGRRRRRRRRRRRR, was added to each protein C-terminal. The final DNA fragment was flanked with *Nde* I and *Xho* I sites, and inserted into pET41a expression vector *Nde* I–*Xho* I sites for protein expression. Each plasmid was verified by DNA sequencing.

Protein expression and purification

The above protein expression plasmids were individually transformed into BL21 (DE3) competent cells, and the recombinant protein production was carried out using auto-induction method. Briefly, proteins were expressed in cells by induction with 0.5 mM isopropyl-1-thio-β-Dgalactopyranosid. The cells were then collected by centrifugation, and the pellets were subjected to freeze-and-thaw cycles. The inclusion bodies released were first washed extensively with a buffer containing 8 M urea, 100 mM Tris, 1 mM glycine and 10 mM ßMercaptoethanol (pH8). The solubilized inclusion bodies were refolded with a rapid dilution method as described previously (Hou et al., 2005; Lafevre-Bernt et al., 2008; Medynski et al., 2007). The refolded protein was concentrated using tangential flow filtration and purified by size exclusion chromatography using a Superdex-200 column (XK26x850-mm, GE, Piscataway, NJ), and confirmed using SDS-PAGE.

Western blotting

We used Nanodrop ND-1000 to measure purified protein concentration (A280). Protein samples were separated on Novex® 4-20% Tris-Glycine Gel and blotted onto nitrocellulose membrane (GE, USA). For identification of recombinant proteins, Oct3/4 (sc-5279; Santa Cruz Biotechnology, USA), Sox2 (AP2048d; Abgent, USA), Klf4 (AF3640-pu, R&D, USA) and cMyc (AF3696, R&D, USA) antibodies were used. Anti-mouse, anti-rabbit and anti-goat HRPconjugated IgGs (Cell Signalling Technology) were used as secondary antibodies. Signals were detected using SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific, USA).

Cell culture

OG2-MEFs were cultured on gelatin-coated dishes in normal MEF media: high-glucose D-MEM (Invitrogen) with 10% FBS, 0.1 mM non-essential amino acids, and 2 mM L-glutamine. piPS cells were cultured on irradiated CF1 MEFs with normal mESC growth media, which consist of Knockout DMEM (Invitrogen) supplemented with 20% KSR, 0.1 mM 2-ME, 2 mM L-glutamine, 0.1 mM non-essential amino acids, and 10^3 units/ml LIF (ESGRO, Chemicon International). The piPS cells were passaged every 3 days as a single cell suspension using 0.05% trypsin/EDTA and seeded at 1.0×10^4 cells per cm^2 for routine culture. For feeder-free culture, cells are grown on gelatin-coated tissue culture dishes in chemically defined media, which consist of Knockout DMEM supplemented with 1xN2, 1xB27, 0.1 mM 2-ME, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 50 µg/ml BSA fraction V (GIBCO), 10^3 units/ml LIF and 10 ng/ml BMP4 (R&D).

Generation of piPS cells

OG2-MEFs were seeded at 5×10^4 cells per well in a 6-well plate coated with gelatin in normal MEF media (DMEM supplemented with 10% FBS). On the next day, media was changed to the protein transduction media, which were prepared by mixing the recombinant reprogramming proteins at the final concentration of 8 μg/ml with regular mES cell growth medium supplemented with 10^3 units/ml LIF. After overnight culture in the protein transduction media, media was changed to normal mESC growth media, and cells were cultured for additional 36 hours before repeating the same protein transduction cycle. Total four protein transduction cycles were applied on the cells. After completing protein transduction, cells were then passaged onto irradiated CF-1 MEF feeder cells at day 9 in normal mESC growth media. Media were changed every 3-4 days until GFP^+ colonies were observed around day 30-35. GFP^+ colonies were then passaged onto new irradiated MEF feeder cells in normal mESC growth media, and stably maintained and expanded as piPS cells. Some colonies were further selected and expanded in the presence of pluripotin (1 μM) or PD0325901 (1 μM).

Spontaneous in vitro differentiation

The pluripotency of piPSCs were examined by in vitro differentiation from embryoid bodies (EBs). piPSCs were trypsinized into single cells and cultured in suspension on low adhesion plates (Corning) in DMEM medium supplemented with 10% FBS. Media were refreshed every other day and EBs were allowed to grow for 6 days in suspension. EBs were then replated onto 0.1% gelatin-coated plates. Spontaneous differentiations were examined by immunostaining or RT-PCR of representative lineage specific markers with indicated antibodies or primers at various time points (3 up to 16 days).

Cytochemistry and immunofluorescence assay: ALP staining was performed using the Alkaline Phosphatase Detection Kit (Chemicon) as instructed by the manufacturer. Immunocytochemistry was performed using standard protocol. Briefly, cells were fixed in 4% paraformaldehyde (Sigma-Aldrich), washed three times by PBS, and then incubated in PBS containing 0.3% TritonX-100 (Sigma-Aldrich) and 5% normal donkey serum (Jackson Immuno Research) for 1 hr at room temperature. The cells were then incubated with primary antibody at 4°C overnight: Albumin (Abcam, AB19188, 1:200); Brachyury (Santa Cruz, C-19, 1:200); Cardiac troponin t antibody (CT3) (Developmental Studies Hybridoma Bank, 1:700); Gata4 (Santa Cruz, H-112, 1:300); MAP2ab (Abcam, ab5392, 1:1000); MF20 (Developmental Studies Hybridoma

Bank, 1:200); Nanog (Abcam, ab21603, 1:500); Oct4 (Santa Cruz, sc-5279, 1:100); Pax6 (Developmental Studies Hybridoma Bank, 1:2000); Pdx1 (Millipore, AB3243, 1:500); Sox2 (Millipore, AB5603, 1:500); Sox17 (R&D systems, AF1924, 1:300); SSEA1 (Santa Cruz, sc21702, 1:100); Tuj-1 (Covance, MMS-435P, 1:1000). After washing three times with PBS, cells were incubated with secondary antibodies: Alexa Fluor 555 donkey anti-mouse IgG (1:2000, Invitrogen), Alexa Fluor 555 donkey anti-goat IgG (1:2000, Invitrogen), Alexa Fluor 555 donkey anti-chicken IgG (1:2000, Invitrogen),or Alexa Fluro 555 donkey anti-rabbit IgG (1:2000, Invitrogen) for 2 hr at RT. Nuclei were detected by DAPI (Sigma) staining. Images were captured by Zeiss HXP 120.

Semi-quantitative RT-PCR

Total RNAs were extracted using RNeasy plus mini kit (Qiagen), reverse transcribed with iScript cDNA Synthesis Kit (BioRad) according to manufacturers' instructions. PCR products were resolved on (1.5 %) agarose gels and visualized by ethidium bromide staining. Images were taken using Bio-Rad Gel document system. Primers used are listed in Supplemental Table S1.

Bisulfite sequencing analysis

DNAs from R1 cells, OG2 MEFs, and piPS cells (passage 9) were isolated using the Non Organic DNA Isolation Kit (Millipore). The DNAs were then treated for bisulfite sequencing with the EZ DNA Methylation-Gold Kit (Zymo Research Corp., Orange, CA). The treated DNAs were then used to amplify sequences of interest. Primers used for promoter fragment amplification were as previously published (Blelloch et al., 2006) and listed in Supplemental Table S1. The resulting fragments were cloned using the TOPO TA Cloning Kit for sequencing (Invitrogen) and sequenced.

Microarray analysis

The Illumina Sentrix BeadChip Array MouseRef-8 v2 (Illumina, CA, USA) was used for microarray hybridizations to examine the global gene expression of murine ES cells, piPS cells and OG2-MEFs. Biotin-16-UTP-labeled cRNA was synthesized from 500 ng total RNA with the Illumina TotalPrep RNA amplification kit (Ambion AMIL1791, Foster City, CA, USA). The hybridization mix containing 750 ng of labeled amplified cRNA was prepared according to the Illumina BeadStation 500X System Manual (Illumina, San Diego, CA, USA) using the supplied reagents and GE Healthcare Streptavidin-Cy3 staining solution. Hybridization to the Illumina Array MouseRef-8 v2 was for 18 h at 55 °C on a BeadChip Hyb Wheel. The array was scanned using the Illumina BeadArray Reader. All samples were prepared in two biological replicates. Processing and analysis of the microarray data were performed with the Illumina BeadStudio software. The data were subtracted for background and normalized using the rank invariant option.

Chimera formation

piPS cells were aggregated with denuded post-compacted eight-cell stage embryos to obtain aggregate chimeras. Eight-cell embryos were flushed from females at 2.5 dpc and cultured in microdrops of KSOM medium (10% FCS) under mineral oil. Clumps of piPS cells (10-20 cells) after short treatment of trypsin were chosen and transferred into microdrops containing zonafree eight-cell embryos. Eight-cell embryos aggregated with piPS cells were cultured overnight at 37

°C, 5% CO_2. Aggregated blastocysts that developed from eight-cell stage were transferred into one uterine horn of a 2.5 dpc pseudopregnant recipient.

Table S1. Primers used for PCR

Gene	Forward	Reverse
For RT-PCR		
AFP	ACAGGAGGCTATGCATCACCAGTT	TGCTCCTCTGTCAGTTCAGGCTTT
Brachyury	ATGCCAAAGAAAGAAACGAC	AGAGGCTGTAGAACATGATT
cMyc	CAGAGGAGGAACGAGCTGAAGCGC	TTATGCACCAGAGTTTCGAAGCTGTTCG
E-cad	GCAGTCAGATCTCCCTGAGTTCGAG	CTACATACAAAGGTCACTCTAGCAAC
FoxA2	AAGGGAAATGAGAGGCTGAGTGGA	ATGACAGATCACTGTGGCCCATCT
GAPDH	GTGTTCCTACCCCCAATGTGT	ATTGTCATACCAGGAAATGAGCTT
Klf4	CACCATGGACCCGGGCGTGGCTGCCAGAAA	TTAGGCTGTTCTTTTCCGGGGCCACGA
Nanog	AGGGTCTGCTACTGAGATGCT	CAACACCTGGTTTTTCTGCCACCG
Oct4	CTGAGGGCCAGGCAGGAGCACGAG	CTGTAGGGAGGGCTTCGGGCACTT
Otx2	CCGACTTTGCGCCTCCAAACAA	GGTTGATGGACCCTTCTAAGGC
Pax6	GCTTCATCCGAGTCTTCCCGTTAG	CCATCTTGCTTGGGAAATCCG
Pdx1	CTCCGCCGCCACCCCAGTTTAC	GCGGGGCCGGGAGATGTATTTG
Rex-1	TGAAAGTGAGATTAGCCCCGAG	GTCCCATCCCCTTCAATAGCAC

Sox1	CCTCGGATCTCTGGTCAAGT	TACAGAGCCGGCAGTCATAC
Sox2	GGTTACCTCTTCCTCCCACTCCAG	TCACATGTGCGACAGGGGCAG
Sox17	TGCCCTTTGTGTATAAGCCCGAGA	GGGTAGTTGCAATAGTAGACCGCT
For bisulfite-sequencing PCR		
Oct4	GTTGTTTTGTTTTGGTTTTGGATAT	CCACCCTCTAACCTTAACCTCTAAC
Oct4	ATGGGTTGAAATATTGGGTTTATTTA	CCACCCTCTAACCTTAACCTCTAAC
Nanog	GAGGATGTTTTTTAAGTTTTTTTT	CCCACACTCATATCAATATAATAAC
Nanog	AATGTTTATGGTGGATTTTGTAGGT	CCCACACTCATATCAATATAATAAC

Figure S1. Characterization of recombinant reprogramming proteins.

(A) Schematic of the protein expression vector. (B) Following protein refolding and purification, Oct4 (lane 2), Sox2 (lane 3), Klf4 (lane 4), and cMyc (lane 5) were analyzed by 4-12% Bis-Tris NuPage Gel and Coomassie blue staining. The protein standards were shown in lane 1. (C) Stability of the four recombinant reprogramming proteins under the cell culture condition was examined by Western blot analysis. Proteins (8 μg/ml) were added into mESC growth media and incubated at 37 °C for 12 hr. Medium samples were then collected and subjected to Western blot analysis. The specific antibodies against Oct4, Sox2, Klf4, and cMyc were used. (D) Protein transduction of 11R-tagged reprogramming proteins into MEF cells was examined by immunocytochemistry. Eight μg/ml of Oct4 (GREEN), Sox2 (RED), Klf4 (RED) and cMyc (GREEN) proteins were added to OG2-MEF cells. Cells were cultured for 6 hr, 24 hr and 48 hr, and then fixed and immunostained. Cells were also stained with DAPI to visualize the nuclei (BLUE) and the images were merged.

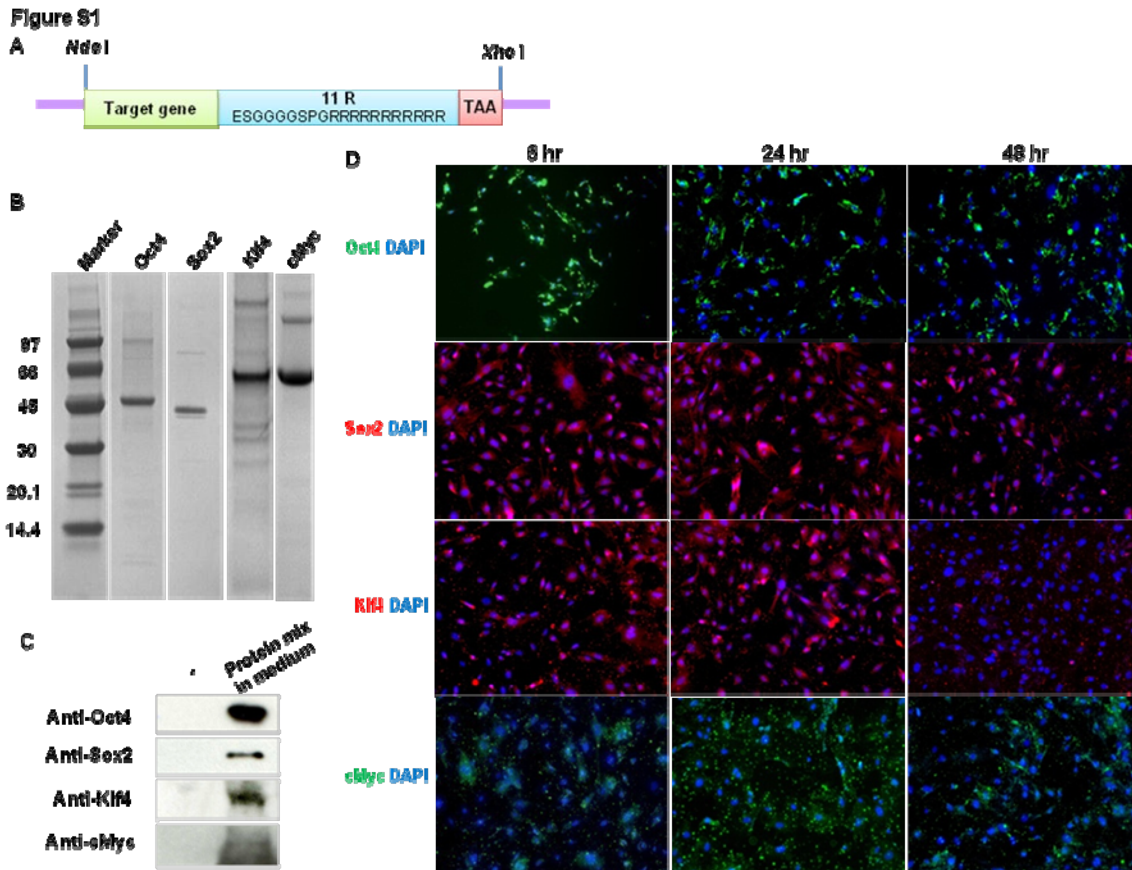
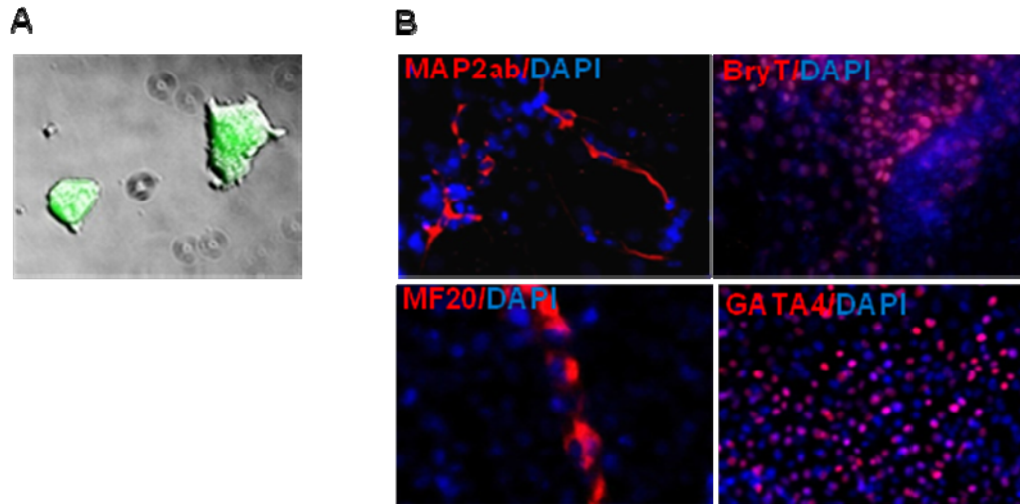


Figure S2. Additional characterizations of piPS cells. (A) piPS cells clonally expand and self-renew in chemically defined media and feeder free condition. (B) piPS cells can effectively differentiate *in vitro* into mature neurons (MAP2ab^{+}), BryT^{+} mesoderm cells, mature cardiomyocytes (MF20^{+}), and GATA4^{+} endoderm cells. (C) GFP genotyping of chimeric embryos. Five fetuses were randomly picked, including one fetus which showed Oct4-GFP positive cells in the gonad. GFP integration in five different tissues, namely heart, liver, brain, tail and gonad, was analyzed by genomic PCR. Positive detection of GFP sequence in all five tissues of 3 fetuses, in

four tissues of one fetus, and in three tissues of one fetus, confirmed that piPS cells could contribute to the three germ layer (mesoderm, endoderm and ectoderm) plus gonad *in vivo*.

Figure s2



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Summary of GFP genotyping

	Heart	Liver	Brain	Tail	Gonad	Germline contribution
Embryo-9	+	+	+	+	+	+
Embryo-1	+	+	+	+	+	-
Embryo-6	+	-	+	+	-	-
Embryo-7	+	+	+	+	+	-
Embryo-13	+	+	-	+	+	-

Supplemental References

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